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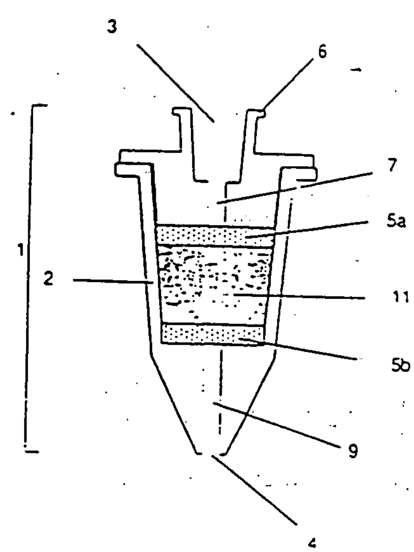
## (54) Method for separating long-chain nucleic acids.

(57) Long-chain nucleic acids are separated from other substances from solutions containing nucleic acids and other materials, and more particularly nucleic acid/protein mixtures from biotechnical preparations from bacteria, viruses, animal and vegetable tissues and cells as well as body liquids, more particularly cell ingredients and/or degradation products thereof as well as components of body liquids which components are not long-chain nucleic acids, by that the long-chain nucleic acids in the nucleic acid-containing solutions, or after disintegration under mild conditions of the tissue cells and/or cells from body liquids are fixed on a porous matrix, whereas the substances to be separated therefrom are washed out from the matrix, and the fixed nucleic acids, if desired, are subsequently removed from the matrix. A device for carrying out the method preferably consists of a cartridge (1) containing the porous matrix (11) and having at least one inlet opening (3) and at least one outlet opening (4).



# METHOD FOR SEPARATING LONG-CHAIN NUCLEIC ACIDS

The present invention relates to the separation of long-chain nucleic acids from other substances from solutions containing nucleic acids and other materials, and more particularly nucleic acid/protein mixtures from biotechnical preparations from bacteria, viruses, animal and vegetable tissues and cells, more particularly cell ingredients and/or degradation products thereof as well as components of body liquids which components are not long-chain nucleic acids, and to the device for carrying out the method.

The preparation of nucleic acid from natural sources, and more particularly from viruses, bacterial and eucaryotic cells, cell aggregates or tissues as well as body liquids is a key technique for various preparative and analytical problem solutions in biology and medicine. Some important applications may be mentioned by way of example hereinafter:

Molecular biology uses vehicles capable of replicating for DNA fragments which include plasmides, phages, viruses etc.. In order to be able to use the DNA-or RNA-processing enzymes, first a highly purified DNA or RNA is needed. The same is applicable to genetical analytics of, for example, viruses from tissue liquid or genomic DNA from tissue. Since for a specific detection of certain characteristics of nucleic acids such as, for example, restriction polymorphisms, said nucleic acids prior to analysis are subjected to an enzymatic degradation, they must be present in such a purity that these methods of enzymatic are usable. The methods so far known do not allow to extract and to concentrate the DNA/RNA by following similar and simple instructions for operation from starting materials being so different as solutions containing nucleic acids and other materials, more particularly nucleic acid/protein mixtures from a biotechnological preparation, tissue, blood, sputum, cell cultures, bacteria, fungi, renal and fecal excrements.

The problems will become more clearly evident in consideration of virus diagnostics, for example the detection of Hepatitis B-DNA in blood and liver biopsies, the individual assignment in criminialistics, forensic medicine or paternity analysis, wherein the analytical methods to be employed require cellular nucleic acids from very different sources such as sperms, tissue (fresh, carbonized, frozen, dried etc.) for use in the technically comparable kind of subsequent analysis.

The methods as so far known for the purification of long-chain nucleic acids require centrifugation steps of extended duration or aqueous phenol/two-phase extractions. Such procedures are rather intensive in personnel and equipment cost and, moreover, too expensive to be simply realizable in an automated operation. Furthermore, the known and conventionally used purification methods involve the use of expensive equipment such as cooled centrifuges and ultracentrifuges which, in addition, consume valuable materials such as cesium chloride for density gradient centrifugation and rotor insertions for one-time use.

A method described in EP-A-0 104 210 and based on the use of HPLC devices is suitable for a chromatographic separation of nucleic acids; however, long-chain nucleic acids such as, for example, λ-phage DNA, are damaged by the mechanical action.

From Bernardi, G. (1971), "Methods in Enzymology" (Grossman, L. & Moldave, K., Edit.) Vol. 21, pages 95 to 139, Academic Press, New York, there has been known a method for the separation of nucleic acids from proteins, lower molecular weight substances and cellular components such as oligo-and polysaccharides by chromatographic purification on hydroxylapatite (HAP). This method has also been used for the purification of plasmides and λ-phage DNA (cf. Colman, A. et al. 1978, Eur. J. Biochem. 91, 303 to 310; Shoyab, M. & Sen, A., 1978, J. Biol. Chem. 253, 6654 to 6656; and Johnson, T.R. & Ilan, J., 1983, Anal. Biochem. 132, 20 to 25). However, this method is not comparable to the method according to the invention. Thus, for example, the separation efficiency, expressed in milligrams of nucleic acid per grams of separating gel, which amounts to about 1 mg/1 g in the method according to the invention is about 100 times higher than that of the HAP method. For long-chain nucleic acids the separation on HAP results in high losses in yield, more specifically of cellular DNA, and requires high phosphate and urea concentrations in the eluting buffer, which adversely affects further processing of the separated long-chain DNA. The known gel permeation procedures are not capable of separating high molecular weight nucleic acids from other high molecular weight substances such as proteins and polysaccharides, since these materials will only select by size and shape.

For the direct hybridization reaction the product purity as obtained by known methods is usually sufficient. However, for a number of detection problems the concentration of the purified long-chain nucleic acid is too low for allowing direct detection by hybridization.

As examples there may be mentioned the analysis of AIDS virus nucleic acids in much under-represented infected cells of a lymph node biopsy or the detection of a restriction fragment length

polymorphism (RFLP) in a small amount of cells obtained upon an amniocentesis or chorion biopsy.

If specific nucleic acid sequences are to be enzymatically amplified, then the nucleic acid to be amplified must be present in such a purity that enzymes such as polymerases will not be inhibited (Saiki, R.K. et al., 1985, Science 230 1350 to 1354). An essential purification step of the known methods is the use of a phenolic extraction in order to efficiently effect the removal of proteins and organic agents which may inhibit enzymes. However, phenol is a strong poison to skin and liver and should be processed only by well trained staff unter strict precautions. Moreover, liquid extractions are time-consuming and intensive in personnel.

So far such purifications of long-chain nucleic acids, more particularly in molecular biology, could be carried out only in research institutes, for the known methods are time-consuming and intensive in instrumentation and cost and, moreover, due to the used chemicals is dangerous to health. A typical instruction for operation may be classified into the following steps:

a) the disintegration and digestion of the cells or tissues or body liquids for which a number of methods may be employed such as mechanical methods (for example milling) in combination with other physical methods (for example a boiling procedure - "Koch-Verfahren"), with enzymatic methods (using, for example, proteinase K, lysozyme etc.) and with chemical methods (using, for example, sodium hydroxide solution, diethyl pyrocarbonate) and which renders the cell contents accessible to further enzymes and reagents;

b) a coarse clarification of the solution from cell debris by means of a centrifuge;

c) steps for the removal of proteins and first accumulation of the nucleic acid, usually by utilization of a two-phase system consisting of phenolic phase/aqueous phase; and

d) high purification techniques such as ultracentrifugation.

The known methods for purifying long-chain nucleic acids ( > 20 kB ≙ molecular weight > 13 million Dalton) have in common that they are difficult to rationalize if the nucleic acid preparations are to be carried out as routine operations. Such condition, for example, exists in laboratories of molecular biology which permanently have to provide highly pure plasmides or phage DNA.

In medical diagnostics there is an urgent demand to obtain new information and knowledge from the analysis of genetic material. Hereto, the problem of hepatitis diagnostics may be mentioned, where only the direct detection of the virus will provide information on the infectiosity, or the genetic detection of a genetically caused protein deficieny, for example of a thalassemia. The work-up of the material to be analyzed (DNA or RNA), more particularly with large sample numbers, has proven to be crucial barrier on the route to a genetics-based diagnostics. if the latter should match the known serologic methods with respect to the applicability thereof to large sample populations.

The importance of an automated nucleic acid work-up is extremely high. This mode of operation is a pre-requisite for a generally applicable genetics-based diagnostics which with respect to the importance thereof could correspond to the widely used methods of serologic diagnostics. Both methods cover areas, the respective information obtainable from which will add up to each other in a complementary manner.

While in immunology the cell or virus products could be qualitatively and quantitatively determined, by genomic analysis the diagnosis is verified on the level of the information store of the nucleic acid.

Gene technology enables an extremely high-resolving diagnostics to be effected due to the fact that nearly each individual structural element of the genetic store comprising up to billions of structural elements can be examined. The procedure allows to determine the presence or absence of infectious genetic material, for example of an AIDS causationist, or to recognize genetic diseases such as muscle dystrophia without gene products having to be expressed, for example in the form of protein/antigens, or the absence thereof having to be determined.

Furthermore, biotechnology, and more specifically gene technology, enables products to be produced by means of transformed microorganisms. However, under this aspect there arises a very serious problem from that it cannot be excluded that upon use of products having been prepared by biotechnology potentially noxious genetic information is taken over into the cell or into the genetic information, respectively, of the user and there cause transformation, infection, resistance to antibiotics etc. to occur.

The problem is all the more serious as recently increasedly homologous transformed cell systems are employed in the place of organisms such as E. coli. or yeast, such as, e.g., hamster ovar cells, human fibroblasts, cancer cells etc .. This is pursuant to the goal, if possible, to produce human-identical protein products which with respect to conformation and, above all, modification such as glycosylation and other post-translational modifications are equal to human proteins.

However, simultaneously therewith the danger is enhanced of a possible transformation of human cells of a patient by homologous DNA sequences

or adapted vector systems having their properties such as self-reproducibility, resistance behavior, presence of strong promotors, enhancer elements, oncogenetic information such as "gene dose" effects. Such apprehensions were uttered also with respect to genetic information obtained from E. coli, yeast, B. subtilis etc.. Thus, the danger exists of that preparations contain nucleic acid which either directly acts as a pathogen, such as in the case of certain viruses and of oncogenetic DNA, or which may indirectly act to initiate a cancer by becoming integrated in the receptor DNA and initiating mutations thereupon. This is why it is desirable that all therapeutic products as much as possible are free from nucleic acids. Thus, the American health authorities {Food and Drug Administration (FDA)} for the time being recommends that not more than a dose of 1 to 10 pg/day of DNA should be administered.

Upon application of the methods according to prior art, depending on the kind of producing system, in the first steps of purification of the biotechnically prepared products varying amounts of nucleic acid are obtained. Only traces of contaminating nucleic acid are present once the celles continuously secrete the synthetized product so that only undesiredly lyzed cells will significantly release nucleic acids. However, the total cell equivalent of nucleic acids may also occur as contamination, if after batch production the host cells are completely lyzed for intended product release or being killed. In this latter case the first step frequently is the precipitation of the DNA/RNA by polycations such as polyimine. However, this step does not lead to a complete removal of the substances.

Therefore, it is the object of the present invention to provide a process for removing long-chain nucleic acids from tissues and body liquids which

a) in a similar manner allows the nucleic acids to be extracted and concentrated from the most various starting materials such as tissue, blood, sputum, cell cultures, bacteria, fungi, renal and fecal excrements as well as vegetable tissue from callus cultures, roots etc.,

b) requires no long-time centrifugation steps, and more specifically no ultracentrifugation,

c) can be carried out without expensive equipment, and more specifially without refrigerated centrifuges and ultracentrifuges, and without using valuable material such as cesium chloride for density gradients or rotor insertions for one-time use,

d) ensures high purity of the nucleic acid to be attained,

e) works without phenolic extraction step, and

f) is suitable for being automated, and by means of extraction of the long-chain nucleic acid separates mixtures of long-chain nucleic acids and other materials such as those obtained when products are biotechnologically produced.

In the EP-A-0 104 210 there has been described a method for separating nucleic acids up to plasmide size ( < 10 000 base pairs ≙ 6 million Dalton). By using the material described therein which is distinguished by that a highly porous silicagel provided with an anion exchanger coating and employed in HPLC chromatography is used as a carrier, for example, pre-purified plasmides may be prepared in a highly pure state. Nevertheless, here also centrifugation steps and precipitation steps are necessary which are not suitable for application in bulk analysis and preparation, respectively. One crucial drawback consists of that for larger molecules, for example λ-phage DNA, during the chromatographic separation of particles < 10 µm the shear forces become so high that intact molecules cannot be recovered any more. This is all the more applicable to cellular DNA having the multiple length of λ-phage DNA.

The object of the present invention is attained by a method wherein the long-chain nucleic acids from bacteria cells, viruses, vegetable and animal tissue cells and/or cells from body liquids after disintegration under mild conditions or from mixtures containing nucleic acids and other materials, more specifically nucleic acid/protein mixtures from a biotechnical preparation, are fixed on a porous matrix, whereas the substances to be separated therefrom are washed out from the matrix, and the fixed nucleic acids are subsequently removed from the matrix.

The porous matrix preferably consists of a material for chromatography on the base of silicagel, diatomite, aluminum oxide, titanium dioxide, hydroxylapatite, dextran, agarose, acrylamide, polystyrene, polyvinyl alcohol or other organic polymers, derivatives or of copolymers of the above-mentioned carrier materials, the surfaces of which preferably have been modified, more specifically with chemical groups exibiting anion exchanger activities.

In a particularly preferred embodiment the porous matrix consists of modified silicagel particles having a particle size of from 15 to 250 µm, and preferably from 25 to 40 µm. The pores have diameters of from 100 to 2,500 nm, and preferably of about 400 nm. The modification of the silicagel is preferred to be effected by reacting the carrier material to form a silanating reagent to form an anion exchanger.

As has been disclosed in the EP-A-0 104 210 this reaction employs γ-glycidyloxypropyl trimethoxysilane and N,N-dimethylaminoethanol as

reactants.

The process according to the invention, inter alia, makes it possible to avoid a phenolic extraction of the digestion mixture for purifying the long-chain nucleic acids from interfering components.

In the process according to the invention it is recommended to use hydrophilic surfaces, since nucleic acids, and more particularly long-chain nucleic acids, tend to strongly interact with the matrix, if salt solutions of high ionic strength are used. The strong hydrophobic interactions may give rise to contamination and yield problems.

The mild enzymatic proteolysis may be carried out either alone or in combination with the application of mechanical means. A number of methods are available, namely mechanical methods (for example milling) in combination with other physical methods (for example a boiling procedure - "Koch-Verfahren"), enzymatic methods (using, for example, proteinase K, lysozyme etc.) and chemical methods (using, for example, sodium hydroxide solution, diethyl pyrocarbonate).

These methods may be employed either alone by themselves or in combination with the method according to the invention for the extraction of long-chain nucleic acids. Some of these known methods {T. Maniatis, E.F. Fritsch, J. Sambrook (CSH), 1982, "Molecular cloning" (C.S.H.)} utilize sodium dodecylsulfate (SDS) or Sarcosyl® as detergent or solubilizing and protein-denaturing agent. In the presence of more than 0.1% of SDS (preferred to be used are from 0.1 to 2%) the bond of DNA/RNA to the polycationic surface of the carrier is affected and greatly reduced. If a use of SDS is inevitable for the digestion, then the aqueous phase must be admixed with phenol and/or chloroform, i.e. a liquid-liquid extraction is necessary in order to remove the SDS. An alternative is constituted by a step of solid phase extraction by means of hydrophobically coated carriers (reversed-phase carriers) prior to employing method according to the invention.

In the method according to the invention the substances to be separated from the long-chain nucleic acids are removed by thoroughly washing them out with a washing solution of low ionic strength. The eluate formed is virtually free of long-chain nucleic acids. This is particularly advantageous in the removal of long-chain nucleic acids from products having been biotechnologically produced. The method according to the invention allows a separation to be effected of more than 99% up to 100% of long-chain nucleic acids from nucleic acid/protein mixtures.

The porous matrix employed in practicing the method according to the present invention specifically complies with the following criteria which make it particularly useful for removing long-chain nucleic acids from nucleic acid/protein mixtures:

1. High affinity to long-chain nucleic acids;

2. low affinity to other materials, and more particularly to proteins;

3. no unspecific interactions with other materials such as proteins;

4. no unspecific retention of other materials, more specifically of proteins due to inclusions as physically caused (narrow pores);

5. sterilizability;

6. low bleed-off of the porous matrix;

7. no toxic decomposition products of the porous matrix;

8. high capacity of the porous matrix for nucleic acids;

9. regenerability;

10. physiological elution conditions; and

11. high process flow velocity.

The separation of the long-chain nucleic acid from the matrix is effected by rinsing the porous matrix with a solution of high ionic strength (salt concentration).

In the purification of plasmide-DNA, for example from recombinant E. coli bacteria, various methods may be employed for the disintegration of the host cells. All of these methods after centrifugation at about 12.000 g produce the so-called clear lysate, a clear supernatant having been mostly rid of cell debris and chromosomal DNA, which supernatant contains plasmide-DNA, RNA, proteins and other soluble components. Here may be mentioned the lysozyme/Triton method or SDS method, respectively (cf. Maniatis et al.), the NaOH/SDS method (Birnboim, H.C. & Doly, I., 1979, Nucl. Acids Res. 7, 1513 to 1523; Ish-Horowicz, D. & Burke, J.F., 1981, Nucl. Acids Res. 9, 2989 bis 2998), the phenol method (Klein, R.D. et al., 1980, Plasmid 3, 88 to 91) and the "Boiling" method (Holmes, D.S. & Quigley, M., 1981, Anal. Biochem. 114, 193 to 197).

The clear lysates, if they do not contain significant amounts (≤ 0,01%) of ionic detergents such as SDS, they may be directly purified by means of the method according to the present invention, whereby in response to the selection of suitable conditions of ionic strength (preferably from 0.5 to 0.7 M), for example, proteins, lipids, RNA and smaller molecules are separated via adsorption to the porous matrix from long-chain DNA, more specifically DNA from plasmides, the latter materials being bound to the carrier material. The addition of urea to the loading buffer does not affect the binding behavior of the long-chain DNA, while, however, it optimizes the separation efficiency with respect to proteins. Thereby the high capacity of this material of about 1 µg of nucleic acid per 1 mg of the

porous matrix is specifically exploited for DNA in spite of the high molar excess of cellular RNA.

Unspecifically bound RNA and proteins are removed from the porous matrix in few washing steps by washing with buffer solutions of low ionic strength. Then the elution is carried out by extracting the matrix with buffers of high ionic strength.

Due to the unusually high separation efficiency of the method according to the invention between RNA/protein, on the one hand, and long-chain DNA, on the other hand, subsequent RNase, and possible proteinase, treatment(s) as usually employed will not be required. If the clear lysate is SDS-free, such as after a potassium acetate precipitation or as produced by a lysozyme/TritonX-100® lyse, after adjustment of an ionic strength of from 0.5 to 0.7 M the lysate may be directly passed through the porous matrix in order to extract long-chain plasmides. Otherwise, SDS and proteins may first be extracted by phenolization and admixing with chloroform, followed by DNA extraction by means of the process according to the invention. Phenol dissolved in the lysate does not interfere with the plasmide-binding property of the porous matrix.

If the volumes of the lysates are very large, it is recommended first to precipitate DNA with polyethylene glycol (PEG), ethanol or isopropanol. Then the pellet is dissolved in tris-buffer, the solution is adjusted to the desired ionic strength and passed through the porous matrix. Thereby DNS is extracted from the solution, washed with buffers having lower ionic strengths in subsequent washing operations and thereafter re-extracted with tris-buffer of high ionic strength. Then, if desired, DNA may be desalted by a) dialysis, b) precipitation or c) gel permeation chromatography.

The plasmide-DNA isolated by means of the method according to the invention exhibits properties which are at least as good as those of the DNA isolated by using known purification methods. The plasmide DNA may be processed with restriction enzymes and DNA ligases; it is further capable of being sequenced or transfected.

λ-Phages are vehicles frequently used for the transportation of recombinant nucleic acids and are preferred over the plasmide vectors for many applications, as they

a) after protein encapsulation (in vitro packaging) very efficiently introduce alien DNA into cells and, thus, are suitable for establishing comprehensive gene banks;

b) may take in small as well as very large DNA fragments;

c) have good storability;

d) and are easy to cultivate.

Many cloning experiments start with establishing a λ-gene bank, and more specifically a randomly established gene bank. As in this stage only an insufficiently characterized DNA is employed, warranting biological safety is often a problem. Thus, for example, in cloning oncogenetic substances or viral sequences (HTLV-III/LAV-1) safety strains and safety phages of the biological safety level 2 (B2) have to be employed and processed under high laboratory safety conditions (L2 or L3; ZKBS, Berlin; cf. V-th revised version of handling newly re-combined DNA). In the course thereof, work-up steps such as centrifugations, and more particularly elaborate, time-consuming and expensive cesium chloride-gradient centrifugations and harvesting the phages constitute safety problems for laboratory and staff.

The method according to the inventions renders it possible to purify phages /phage-DNA by evading centrifugation steps. A grown or lyzed bacterial culture may be completely worked up, if desired, in a sterile bank to yield λ-DNA having a purity conforming to that of cesium chloride-purified preparations.

Also a single-stranded DNA, for example M13 phage-DNA, can be purified by using the method according to the present invention. From cell lysates single-stranded DNA in high yield and purity may be used for sequencing and hybridization experiments. After the phages have been isolated, the single-stranded DNA is released and adsorbed on the porous matrix. The interfering components are removed by washing.

For an isolation of cellular DNA from tissue of various origin the material is disintegrated and digested using known methods. Thus, a mechanical homogenization, for example under nitrogen, in a ball mill or by efficient maceration and shearing of the material, is followed by a proteolytic digestion in the presence of denaturing and/or solubilizing agents. Proteinase K is a preferred enzyme for the proteolytic digestion, as it efficiently leads to a lysis of cells and cell nuclei even in the presence of 1% of SDS and EDTA. According to prior art, SDS and the proteins have to be removed by time-consuming liquid-liquid extractions, which steps are followed by a dialysis and precipitation of DNA. This procedure is elaborate and difficult to automate. However, the method according to the invention allows to bring long-chain DNA into solution under mild conditions from the sample materials as mentioned above, to fix the DNA on the porous matrix while evading any steps of phenol extraction, and subsequently to elute the DNA under mild conditions in a small volume (0.5 to 5 ml, and preferably 1 to 3 ml).

Infections with viruses play an important role in transfusion and transplantation medicine and generally in immunosuppressed patients. For example, an acute CMV (cytomegalovirus) infection can be detected by an analysis of renal excrements. Ac-

cording to the state of prior art the bacteria are separated from urine by a filtration step or low-speed centrifugation step. and thereafter the virus-DNA is released from the protein sheath and purified by concentration as simultaneously occurring. To this end ultracentrifuges were used in prior art.

The method according to the invention utilizes the described porous matrix by lyzing the CMV viruses in situ by addition of urea, detergent and buffer, whereupon the DNA (130 to 150 $\times$ 10$^6$ Dalton) is released. The DNA is then concentrated by adsorption onto the porous matrix and washed with buffer solutions having low ionic strength. Thereafter the DNA is eluted using a buffer of high ionic strength. If further analysis if followed by a dot-blot technique, it is not required to desalt the DNA.

The use of a porous matrix consisting of a chromatography material on the base of silicagel, diatomite, aluminum oxide, titanium dioxide, hydroxylapatite, dextran, agarose, acrylamide, polystyrene, polyvinyl alcohol or other organic polymers, derivatives or of copolymers of the above-mentioned carrier materials, the surfaces of which preferably have been modified so that the matrix exibits anion exchanger activities, warrants the advantages of the method according to the invention. The particle size of the porous matrix based on silicagel is, for example, 15 to 250 µm, and preferably 25 to 40 µm, and the pore diameter is 50 to 2,500 nm, and preferably about 400 nm.

The device for carrying out the method according to the invention consists of a container made of a material which is resistant to the operation conditions in accordance with the method of the invention. The container receives the porous matrix and has at least one inlet and outlet openings each.

Figure 1 shows schematicly the container, according to a preferred embodiment, for the porous matrix consists of a cartridge 1, which preferably forms a substantially cylindrical hollow body and the side walls 2 of which consist of a material which is resistant to the working conditions (presence of more or less aggressive chemicals and corrosive salts). The side walls 2 preferably are made of a plastics material. Particularly simple is the preparation of the cartridges by using a shrink tube, for example one made of polytetrafluoroethylene (PTFE).

The inlet opening 3 and outlet opening 4 are delimited by filters 5 a and 5 b. In a preferred embodiment the filter consists of a hydrophilic material such as glass, hydrophilic plastics or plastics material coated with a hydrophilic material. However, hydrophobic materials may also be employed. The inlet opening 3 may optionally be shaped so that a Luer Lock system 6 is directly connectable to the inlet cannula. The outlet opening 4, in a preferred embodiment, has an internal tube 8, preferably made of silicone, which is connected to the filter 5 b and preferably does not exceed the end of the outlet cannula 9. The eluate from the cartridge is discharged by a tube 10 which preferably is made of a plastics material, and particularly of a hydrophilic plastics material. Nevertheless, hydrophobic plastics materials such as PTFE may be used as well. The container may also be manufactured by injection molding.

Figure 2 shows schematicly another preferred embodiment of the container according to the invention, which can preferably be produced by means of injection molding. The reference signs have the following meaning:

1 cartridge
2 wall
3 inlet
4 outlet
5a porous fritt
5b porous fritt
7 inlet tube
9 outlet tube
11 porous resin

Figure 3 shows still another preferred embodiment of the container according to the invention. Also this container can be produced by injection molding. The reference signs have the following meaning:

1 cartridge
2 wall
3 inlet
4 outlet
5a porous fritt
5b porous fritt
6 luer-lock connector
7 inlet tube
9 outlet tube
11 porous resin

The internal volume of the container for the porous matrix 11 depends on the intended use. Usually for analytical procedures the internal volume is about 0.02 to 5 cm$^3$, and preferably 0.1 to 1 cm$^3$. If solutions containing nucleic acids and other materials are to be purified on a preparative scale, containers having larger dimensions may be used as well. The porous matrix 11 preferably consists of a silicagel-based anion exchanger. The pore diameter of the material is 50 to 2,500 nm, and preferably about 400 nm, at a particle size of from 15 to 250 µm, and preferably 25 to 40 µm.

The mixture of disintegrated cells from tissue or body liquids, after proteolysis under mild conditions optionally in combination with the application of mechanical means, is introduced into the cartridge via the inlet opening and comes into intimate contact with the porous matrix. Thereupon the matrix extracts the long-chain nucleic acids

from the mixture, whereas the other substance will leave the cartridge via the outlet opening. Attention is to be paid that the applied mixture of the digested material has a low ionic strength. For example, at an ionic strength of about 300 mM of NaCl long-chain RNA and DNA are adsorbed, whereas proteins and lower molecular weight substances are not adsorbed to a significant extent; at concentrations higher than 500 mM of NaCl only long-chain single-stranded DNA and double-stranded DNA are bound, while at salt concentrations around 700 mM NaCl only long-chain double-stranded DNA will be adsorbed on the porous matrix.

Figure 4 demonstrates the typical elution profiles in NaCl-gradient elution at pH 7.0. The absorbance of different substances at 260 nm is plotted versus NaCl concentration. This exemplary diagramm shows the very good separation of the biomolecules. The dotted area symbolizes the range in which proteins, polysaccharides, low molecular weight metabolites and dyes are eluting off the matrix. This happens in the range of from 0 to 0.4 M NaCl. At 0.1 M, for example, are eluting nucleotides, whereas the standard protein BSA (bovine serum albumin) elutes at 0.3 M NaCl concentration. The decamer linker, however, elutes at about 0.4 M NaCl. From the graph it can be taken that tRNA elutes at 0.5 M, 5 S RNA at 0.65 M, 16 S and 23 S rRNA and mRNA between 0.8 M and 0.9 M, M13-phage and other single-stranded (ss) DNA at 1.1 M, double-stranded (ds) DNA of 150 basepairs at slightly below 1.2 M and finally plasmid DNA, for example such of the λ-phage, at 1.3 M NaCl, respectively, the latter one slightly overlapping with the former one. The values are determined only approximately because they might be varying dependent on the experimental conditions as the one skilled in the art expects.

If the porous matrix is synthesized under conditions which do not result in a maximum surface charge density, then the separation profile is altogether shifted to lower ionic strengths, whereas the separa tion efficiency is not significantly affected. This latter effect is even desired if the DNA must be eluted at a lower salt concentration.

After the sample has left the cartridge, the cartridge is carefully rinsed with a washing solution of the desired ionic strength (as set forth above), whereupon the long-chain nucleic acids are desorbed from the porous matrix. This is effected by eluting with a solution of high ionic strength. To this end, in the simplest case the second solution may be introduced through the same inlet opening 3 and be drained through the same outlet opening 4. However, there may also be used cartridges, if desired, which comprise different inlet openings and different outlet openings, respectively, for the solutions having low ionic strength and high ionic strength.

In a further embodiment the method according to the invention may be realized in practice as a "batch" procedure which is distinguished by particularly simple handling. The batch procedure has the advantage of preventing shearing forces to high molecular weight nucleic acids. With this procedure, nucleic acids up to 500.000 basepairs (molecular weight approximately 300 million dalton) can be isolated on a preparative scale without degradation of the shearing force of sensitive molecules. A porous matrix suitable for extracting long-chain nucleic acids is charged in a reaction vessel in a sufficient amount and intimately mixed with the sample to be extracted, with the ionic strength of the solution being adjusted as indicated above. The long-chain nucleic acids are adsorbed on the porous matrix. The contaminating components are removed by several washing steps. Thereafter the longchain nucleic acids are separated under mild conditions from the matrix by elution using a buffer having the desired ionic strength. The porous matrix preferably consists of an anionic exchanger based on a surface-modified chromatography material from silicagel, diatomite, aluminum oxide, titanium dioxide, hydroxylapatite, dextran, agarose, acrylamide, polystyrene, polyvinyl alcohol or other organic polymers, derivatives or of copolymers of the above-mentioned carrier materials. Based on modified silicagel the pore diameter is 50 to 2,500 nm, and preferably about 400 nm, and the particle size of the is 15 to 250 µm, and preferably 25 to 40 µm.

The invention is further illustrated by means of the following examples:

## EXAMPLE 1

The preparation of a plasmide (2860 base pairs) is carried out as follows:

Subsequently to the alkali/SDS digestion procedure a 100 ml culture is centrifuged in LB-ampicillin medium (see Maniatis et al.) with plasmide-transformed HB-101 E. coli cells at 5000 g and 5 °C for 10 minutes. The supernatant is carefully decanted, and the cell pellet is resuspended in 2 ml of 50 mM glucose, 25 mM of Tris-HCl pH 8.0, 10 mM of EDTA.

The sample is allowed to sit at 20 °C for 5 minutes. Then 4 ml of a freshly prepared 1% SDS-solution in 0.2 M NaOH are added thereto and carefully admixed, and the mixture is incubated on ice for 5 minutes. Thereafter, 3 ml of a cold sodium acetate solution (3M Na-acetate, 2M acetic acid) are added thereto and carefully admixed, and the mixture is incubated on ice for another hour. After 10 minutes of centrifugation at about 10000 g, 10

°C a clear plasmide-containing supernatant is obtained. If potassium acetate is used instead of sodium acetate, most of the SDS is precipitated.

In the case of large lysate volumes it is recommended first to precipitate the DNA with PEG, ethanol or isopropanol. Then the pellet is dissolved in 10 mM of Tris-buffer pH 7.5, 1 mM of EDTA, adjusted tp 0.6 M of NaCl and passed over 200 mg of the separating gel. Thereby the DNA (about 100 µg) is extracted from the solution. In the subsequent washing step the gel phase is washed with 0.8 M NaCl, 50 mM of Tris-HCl buffer pH 7.5, 1 mM EDTA and extracted with about 1 ml of 1.2 M NaCl, 50 mM Tris-HCl buffer pH 7.5, 1 mM EDTA. Thereafter the DNA may be desalted by dialysis, precipitation or gel permeation chromatography.

## EXAMPLE 2

The preparation of λ-phage DNA is carried out as follows:

A grown and lyzed λ-phage/E. coli culture (50 ml) is centrifuged at 5000 g and room temperature for 15 minutes or allowed to sit on ice auf Eis for 30 minutes (cf. Maniatis, T. et al.). The supernatants or parts thereof are filtered through narrow-pore sterile filters, for example 0.45 µm, to retain intact cells or floating cell debris.

The suspension of phages is efficiently rid of cellular DNA by passing it through a cartridge (Fig. 1) at an ionic strength of from 0.5 to 0.7 M NaCl. The bed volume of the porous matrix is to be selected so that the capacity is sufficient for the cellular DNA released from the lyzed cells (about 200 mg of porous matrix per 100 ml of lysate).

The filtrate is treated with EDTA (200 mM). Upon simultaneous addition of 4 M of urea the DNA of the phages is released and by means of another filtration through the cartridge specifically adsorbed on the anion exchanger. Then the cartridge is washed with 0.8 M NaCl, 50 mM Tris-HCl buffer pH 7.5, 1 mM EDTA, and the DNA is eluted with about 1 ml of 1.2 M NaCl, 50 mM Tris-HCl buffer pH 7.5, 1 mM EDTA.

The phage DNA thus obtained may be precipitated with PEG or isopropanol. It is also possible to desalt the phage DNA by means of a dialysis (cf. Maniatis, T. et al.). A DNA having high purity is obtained.

## EXAMPLE 3

The preparation of M-13 phage DNA is carried out as follows (an analogous procedure is used for the preparation of single stranded DNA):

The phage lysate is obtained in a conventional manner (cf. EXAMPLE 2) bis disintegration of the cells. After the removal of the cell debris, for example by centrifugation at 5000 rpm for a period of 5 minutes) RNase A is added to a final concentration of 10 µg/ml, and an incubation is allowed to occur at 37 °C for 30 minutes. If the volume of the lysate is too large, a PEG (polyethylene glycol) precipitation of the phage is recommended. 0.3 volumes of 30% PEG and 1.5 M sodium chloride are added and well admixed, and the mixture is allowed to sit on ice for 30 minutes. The precipitated bacteriophage particles are separated from the solution by centrifugation at 10,000 g for 15 minutes. The supernatant is carefully aspirated, and the phage pellet is dissolved in 20 µl of 10 mM Tris, 1 mM EDTA, pH 7.5. Another part by volume of extraction buffer (2 % TritonX-100®, 7 M urea, 100 mM EDTA, pH 7.5) is added, and the mixture is heated at 50 °C for 15 minutes to release the single-stranded DNA.

The cartridge is equilibrated with a buffer of low ionic strength comprising 400 mM sodium chloride, 50 mM MOPS (3-N-morpholino-propanesulfonic acid), 15% ethanol and 1 mM EDTA at pH 7.0. The sample is passed through the cartridge. Then the cartridge is carefully washed with a buffer having a sodium chloride concentration of 750 mM and otherwise a composition as mentioned above. The single-stranded M-13 DNA may be eluted using an elution buffer having a composition of 1.1 M NaCl, 50 mM MOPS, 15% ethanol and 1 mM EDTA at pH 7.0.

## EXAMPLE 4

The isolation of cellular DNA from sperm is carried out as follows:

One hundert µl of sperm are suspended in 1 ml of 500 mM NaCl, 10 mM EDTA, 40 mM DTE, 10 mM Tris-HCl buffer pH 7.5, 1% Triton, 4 M urea and 20 µg/ml of proteinase K and incubated at 37 °C for 2 hours. After centrifugation at about 5000 g for 5 minutes the supernatant is passed through the separating gel in a cartridge. The flow velocity of the supernatant through the cartridge is about 1 ml/min.

Alternatively the "batch" process may be used. In said process the supernatant is intimately mixed with the separating gel by rotation in a 1.5 ml Eppendorf reaction vessel for 15 to 30 minutes. The next step comprises washing the gel five times in the batch process or washing the gel in cartridge with 5 ml of washing buffer (800 mM NaCl, 50 mM Tris-HCl buffer pH 7.5, 1 mM EDTA), followed by the elution with about 1 ml of 1.2 M NaCl, 50 mM Tris-HCl buffer pH 7.5, 1 mM EDTA. The elution yield is higher than 80%. The DNA may further be

desalted by dialysis or precipitation (cf. EXAMPLE 1). The DNA is cuttable with restriction enzymes and is suitable for analysis with the Southern-Blot method (cf. T. Maniatis et al.).

EXAMPLE 5

The preparation of genomic DNA from liver biopsy material is carried out as follows:

Liber biopsy material is mechanically homogenized according to the Potter procedure or any comparable method. To the homogenate proteinase K lysis buffer (cf. EXAMPLE 3), 10-fold volume, is added and the mixture is incubated at 37 °C for 2 hours. The following work-up steps are as described in EXAMPLE 4.

EXAMPLE 6

The preparation of papilloma-virus DNA from verruca biopsy tissue is carried out as follows:

After a mechanical disintegration (liquid nitrogen, ball mill, mechanical squeezing) of verruca biopsy material, in the same manner as described in EXAMPLE 5 ten times the amount of lysis buffer is added, the mixture is incubated at 37°C for 6 hours, and the DNA is worked up as described in EXAMPLE 4. The procedure provides a high molecular weight DNA, which is a mixture of cellular DNA of the human cells and papilloma-virus DNA from the proteolytical digested and lyzed papilloma virions.

EXAMPLE 7

The preparation of CMV (cytomegalovirus) DNA from urine is carried out as follows:

CMV viruses are lyzed in situ upon addition of 4 M urea, 1% Triton, 500 mM NaCl, 50 mM Tris-HCl buffer pH 7.5. The DNA (130 to 150 × $10^6$ Dalton) is released via adsorption on the porous matrix, concentrated in the cartridge shown in Figure 1 and washed as described in EXAMPLE 5. Then the DNA is eluted as described in EXAMPLE 4. Since these operations are usually followed by a Dot-Blot procedure which anyway requires high salt concentrations to be present for binding the DNA to a membrane (nitrocellulose, nylon), the eluted DNA solution is to be adjusted to concentrations of 0.1 M sodium hydroxide and about 2 M sodium chloride. Then a Dot-Blot is directly possible in the devices as conventionally used, for example Minifold I and II by Schleicher & Schüll, West Germany.

EXAMPLE 8

Removal of nucleic acids from protein solutions:

To 5 ml of a BSA (bovine serum albumin) solution (1 mg/ml) there were added 50 ng of pBr 322 plasmide having tetracycline resistance (transformation equivalent about 800 colonies). The obtained solution was adjusted to 0.3 M NaCl to prevent the BSA from being bound to the chromatographie material, and then twice purified over a cartridge containing 250 mg of chromatography material (flow rate 5 ml/hour). Then the cartridge was washed with 1 M NaCl, 50 mM MOPS pH 7.0, and the bound DNA was eluted with 1.5 M NaCl, 15% ethanol, 1 mM EDTA and 50 mM MOPS pH 7.0, precipitated with isopropanol and transformed into E. coli. 800 colonies were counted. The effluent was dialyzed in a parallel operation, and then 100 µl were also transformed. No resistant colonies could be determined. A comparison of the transformation rates of the inital solution and of the eluate allows the conclusion to be drawn that approximately 100% of the DNA present had been removed by using the cartridge.

EXAMPLE 9

Removal of nucleic acids from therapeutic protein preparations:

10 ml of human IgG (5 mg/ml) were traced with 500 pg Eco RI linearized pBR 322 plasmid DNA (10 pg DNA/mg protein). The linearized pBR 322 were labelled with $^{32}P$ to an activity of 5 × $10^5$ counts/min.

The solution was adjusted to 0.3 M NaCl, 0.025 M Na-phosphate, pH 7.0, to prevent binding of the IgG to the chromatographic resin. The protein nucleic acid solution is pumped through a chromatography column (1 cm × 5 cm) filled with 2 g of the anion exchange resin at a flow rate of 1 ml/min. The flow through fraction was collected and the radioactivity was counted.

The column was washed with 50 ml 0.3 M NaCl, 0.025 M Na-phosphate, pH 7.0, at a flow rate of 5 ml/min. The bound nucleic acid was eluted with 1.5 M NaCl, 25 mM Na-phosphate, pH 7. The eluate fraction was collected and precipitated with 1 vol. iso-propanol. The precipitated nucleic acid was dissolved in 0.3 M NaCl, 0.025 M Na-phosphate, pH 7.0 and the radioactivity was counted.

| | |
|---|---|
| Result: Starting activity: | 5.000.000 cpm |
| Flow through fraction: | 15.000 cpm |
| Wash-fraction: | 10.000 cpm |
| Eluate fraction: | 475.000 cpm |

A comparison of the radioactivity led to the conclusion that with this anion exchanger > 95% of the nucleic acids present in a therapeutic protein sample can be removed, reducing the nucleic acid content below 1 pg.

## EXAMPLE 10

Isolation of nucleic acids from protein solutions for analysis of nucleic acid content:

Low nucleic acid contents (≤ 100 pg/ml) in concentrated protein solutions (> 2 mg/ml) cause problems in quantitative analysis of the nucleic acid content of therapeutic protein preparations. For the sensitive analysis by the dot-hybridisation method the protein has to be removed from the nucleic acid. The classical method of proteinase K digestion, phenol/chloroform extraction lead to unreproducible results and loss of nucleic acids and prevent the quantitative analysis.

Extraction with the silicagel based anion exchanger gives a reproducible result, with a recovery of the isolated nucleic acid, without protein contamination, which is suitable for quantitative analysis.

10 mg of mouse IgG of unknown nucleic acid content were dissolved in 5 ml 0.3 M NaCl, 0.025 M Na-phosphate, pH 7.0. A cartridge (0.4 ml) filled with 200 mg silicagel based anion exchanger was equilibrated with 0.3 M NaCl, 0.025 M Na-phosphate, pH 7.0 and the IgG solution was forced through at a flow rate of 0.5 ml/min. The cartridge was washed with 10 ml 0.3 M NaCl, 0.025 M Na-phosphate, pH 7.0, at a flow rate of 2 ml/min. The bound nucleic acid was eluted with 1.5 M NaCl, 0.025 M Na-phosphate, pH 7.0, and precipitated with 0.8 vol. i-prop. The nucleic acid was dissolved in 50 µl 1 mM Tris-HCl, pH 7.0 and the analysis was done by a dot-hybridisation method with a specific mouse-cDNA clone. The efficient binding and quantitative recovery of nucleic acid at the above conditions permit the quantitative analysis of the nucleic acid content in protein solutions even at extremely low nucleic acid contents and very high protein concentrations.

## Claims

1. A method for the separation of long-chain nucleic acids from other substances from solutions containing nucleic acids and other materials, and more particularly nucleic acid/protein mixtures from biotechnical preparations from bacteria, viruses, animal and vegetable tissues and cells as well as body liquids, more particularly cell ingredients and/or degradation products thereof as well as components of body liquids which components are not long-chain nucleic acids, characterized in that the long-chain nucleic acids in the nucleic acid-containing solutions, the tissue cells and/or cells from body liquids after disintegration under mild conditions are fixed on a porous matrix, whereas the substances to be separated therefrom are washed out from the matrix, and the fixed nucleic acids, if desired, are subsequently removed from the matrix.

2. The method according to claim 1, characterized in that the porous matrix consists of a material for chromatography on the base of silicagel, diatomite, aluminum oxide, titanium oxide, hydroxylapatite, dextran, agarose, acrylamide, polystyrene, polyvinyl alcohol or other organic polymers, derivatives or of copolymers of the above-mentioned carrier materials.

3. The method according to anyone of claims 1 or 2, characterized in that the porous matrix is a material for chromatography having been modified with respect to its surface, the material being based on silicagel, diatomite, aluminum oxide, titanium dioxide, hydroxyl apatite, dextran, agarose, acrylamide, polystyrene, polyvinyl alcohol or other organic polymers, derivatives or of copolymers of the above-mentioned carrier materials.

4. The method according to anyone of claims 1 to 3, characterized in that the porous matrix is an anion exchanger.

5. The method according to anyone of claims 1 to 4, characterized in that the particle size of the silicagel-base material is from 15 to 250 µm and the pore diameter is from 100 to 2.500 nm.

6. The method according to claim 5, characterized in that the particle size of the silicagel-base material is from 25 to 40 µm and the pore diameter is about 400 nm.

7. The method according to anyone of claims 1 to 6, characterized in that the separation of the long-chain nucleic acids is carried out with solutions which are phenol-free.

8. The method according to anyone of claims 1 to 7, characterized in that the materials employed have hydrophilic surfaces.

9. The method according to anyone of claims 1 to 8, characterized in that the disintegration under mild conditions is effected by means of an enzymatic proteolysis and/or in the presence of detergents and/or in the presence of denaturing agents or in combination with mechanical procedures.

10. The method according to anyone of claims 1 to 9, characterized in that the substances to be separated are washed out using a washing solution of low ionic strength and the subsequent removal

of the long-chain nucleic acid from the matrix is effected using a washing solution of high ionic strength.

11. The method according to anyone of claims 1 to 10, characterized in that the long-chain nucleic acids are separated from the protein in an amount of more than 99%.

12. The method according to anyone of claims 1 to 11, characterized in that the long-chain nucleic acids are separated from the protein in an amount of up to 100%.

13. The method according to anyone of claims 1 to 12, characterized in that the separation of the long-chain nucleic acids is carried out in a batch process.

14. Use of a porous matrix for the separation of long-chain nucleic acids from other substances from solutions containing nucleic acids and other materials, and more particularly nucleic acid/protein mixtures from biotechnical preparations from bacteria, viruses, animal and vegetable tissues and cells as well as body liquids, more particularly cell ingredients and/or degradation products thereof as well as components of body liquids which components are not long-chain nucleic acids, wherein the long-chain nucleic acids in the nucleic acid-containing solutions, the tissue cells and/or cells from body liquids after disintegration under mild conditions are fixed on a porous matrix, whereas the substances to be separated therefrom are washed out from the matrix, and the fixed nucleic acids, if desired, are subsequently removed from the matrix.

15. Use of a porous matrix according to claim 14, characterized in that the porous matrix consists of a material for chromatography on the base of silicagel, diatomite, aluminum oxide, titanium oxide, hydroxylapatite, dextran, agarose, acrylamide, polystyrene, polyvinyl alcohol or other organic polymers, derivatives or of copolymers of the above-mentioned carrier materials.

16. Use of a porous matrix according to anyone of claims 14 or 15, characterized in that the porous matrix is a material for chromatography having been modified with respect to its surface, the material being based on silicagel, diatomite, aluminum oxide, titanium dioxide, hydroxylapatite, dextran, agarose, acrylamide, polystyrene, polyvinyl alcohol or other organic polymers, derivatives or of copolymers of the above-mentioned carrier materials.

17. Use of a porous matrix according to anyone of claims 14 to 16, characterized in that the particle size of the silicagel-base material is from 15 to 250 µm and the pore diameter is from 100 to 2.500 nm.

18. Use of a porous matrix according to claim 17, characterized in that the particle size of the silicagel-base material is from 25 to 40 µm and the pore diameter is about 400 nm.

19. Use of a porous matrix according to anyone of claims 14 to 18, characterized in that the porous matrix is an anion exchanger.

20. Use of a porous matrix according to anyone of claims 14 to 20, characterized in that the long-chain nucleic acids are separated from the protein in an amount of more than 99%.

21. Use of a porous matrix according to anyone of claims 14 to 20, characterized in that the long-chain nucleic acids are separated from the protein in an amount of up to 100%.

22. A device for carrying out the method according to claims 1 to 12, consisting of a porous matrix in a container having at least one inlet opening and at least one outlet opening.

23. The device according to claim 22, characterized in that the porous matrix consists of a material for chromatography on the base of silicagel, diatomite, aluminum oxide, titanium oxide, hydroxylapatite, dextran, agarose, acrylamide, polystyrene, polyvinyl alcohol or other organic polymers, derivatives or of copolymers of the above-mentioned carrier materials.

24. The device according to anyone of claims 22 or 23, characterized in that the porous matrix is a material for chromatography having been modified with respect to its surface, the material being based on silicagel, diatomite, aluminum oxide, titanium dioxide, hydroxylapatite, dextran, agarose, acrylamide, polystytene, polyvinyl alcohol or other organic polymers, derivatives or of copolymers of the above-mentioned carrier materials.

25. The device according to anyone of claims 22 to 24, characterized in that the porous matrix consists of an anion exchanger.

26. The device according to anyone of claims 22 to 25, characterized in that the container is a cartridge (1).

27. The device according to anyone of claims 22 to 26, characterized in that the cartridge (1) has at least one inlet opening (3) and at least one outlet opening (4).

28. The device according to anyone of claims 22 to 27, characterized in that the side walls (2) of the cartridge (1) consist of a plastics material.

29. The device according to claim 28, characterized in that the plastics material is PTFE.

30. The device according to anyone of claims 22 to 27, characterized in that the side walls (2) of the cartridge (1) consist of a hydrophilic material.

31. The device according to anyone of claims 22 to 28 and 30, characterized in that filters (5a) and (5b) defining the cartridge (1) consist of a hydrophilic material.

32. The device according to anyone of claims 22 to 31, characterized in that the device is manufactured by injection molding.

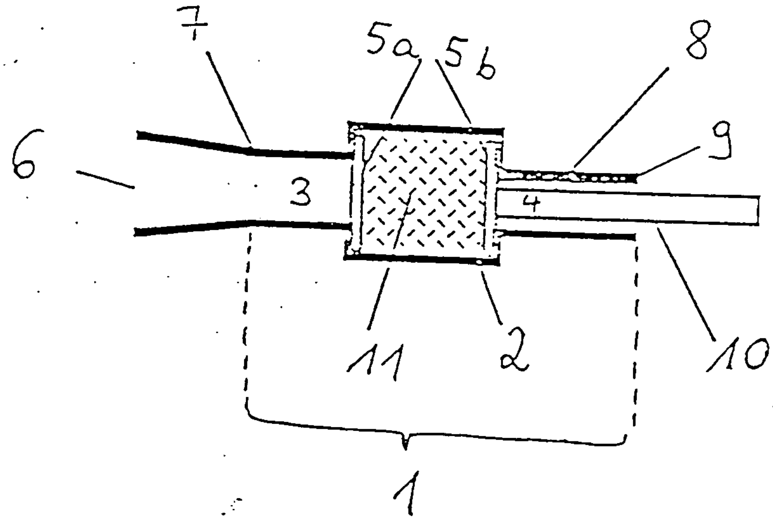



Fig. 1

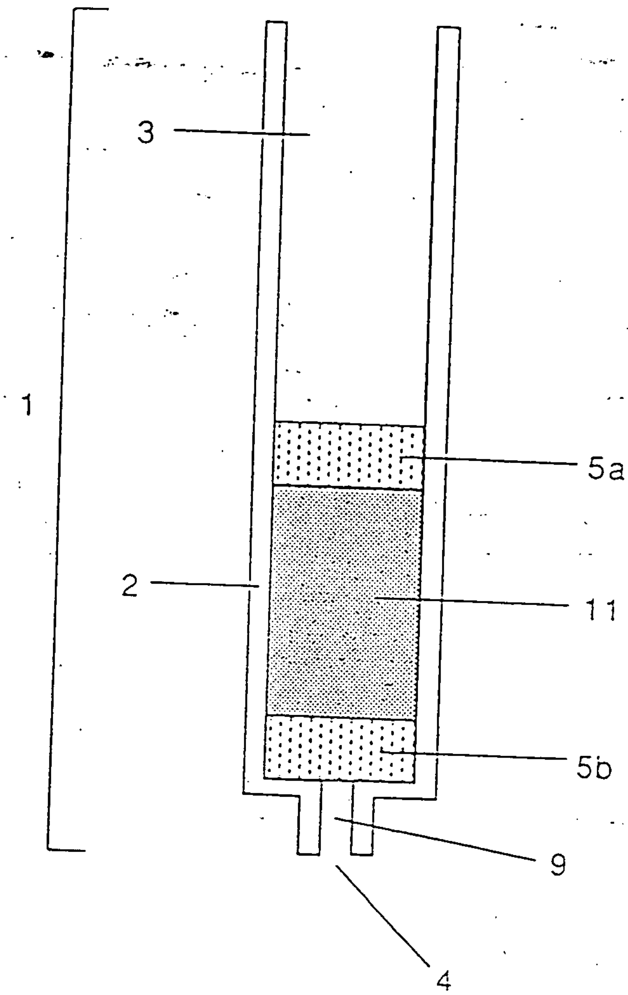



Fig. 2

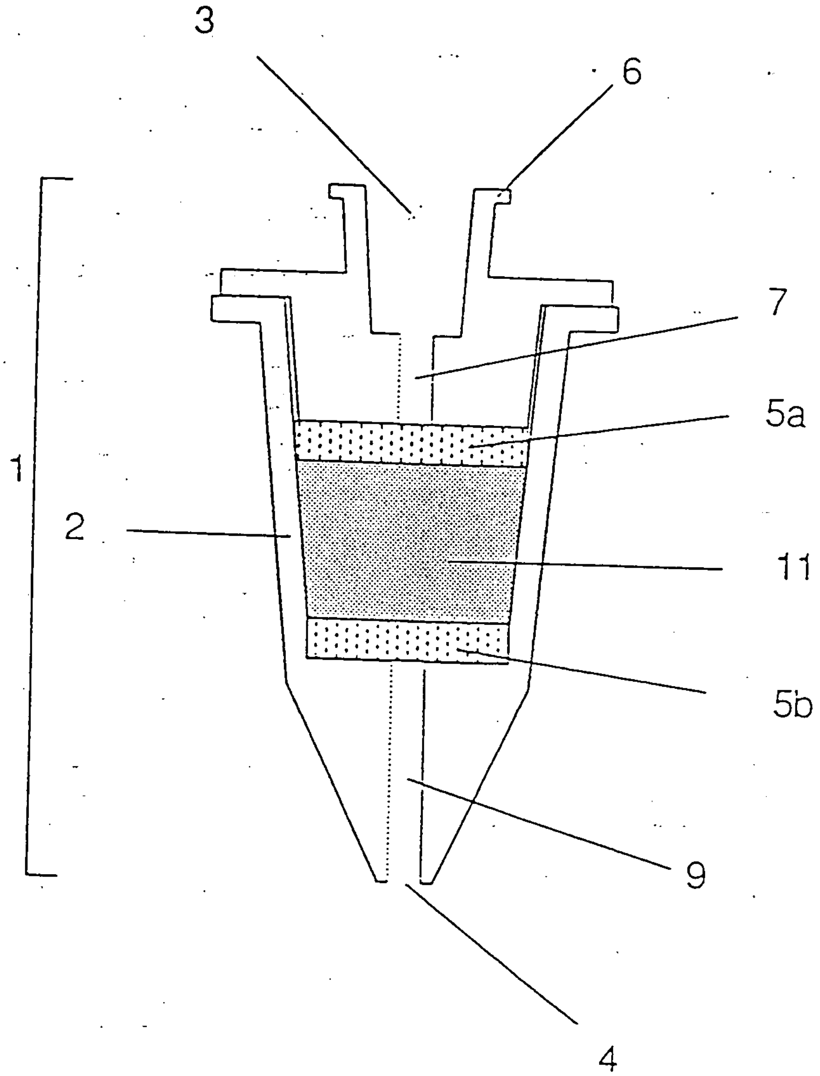



Fig. 3

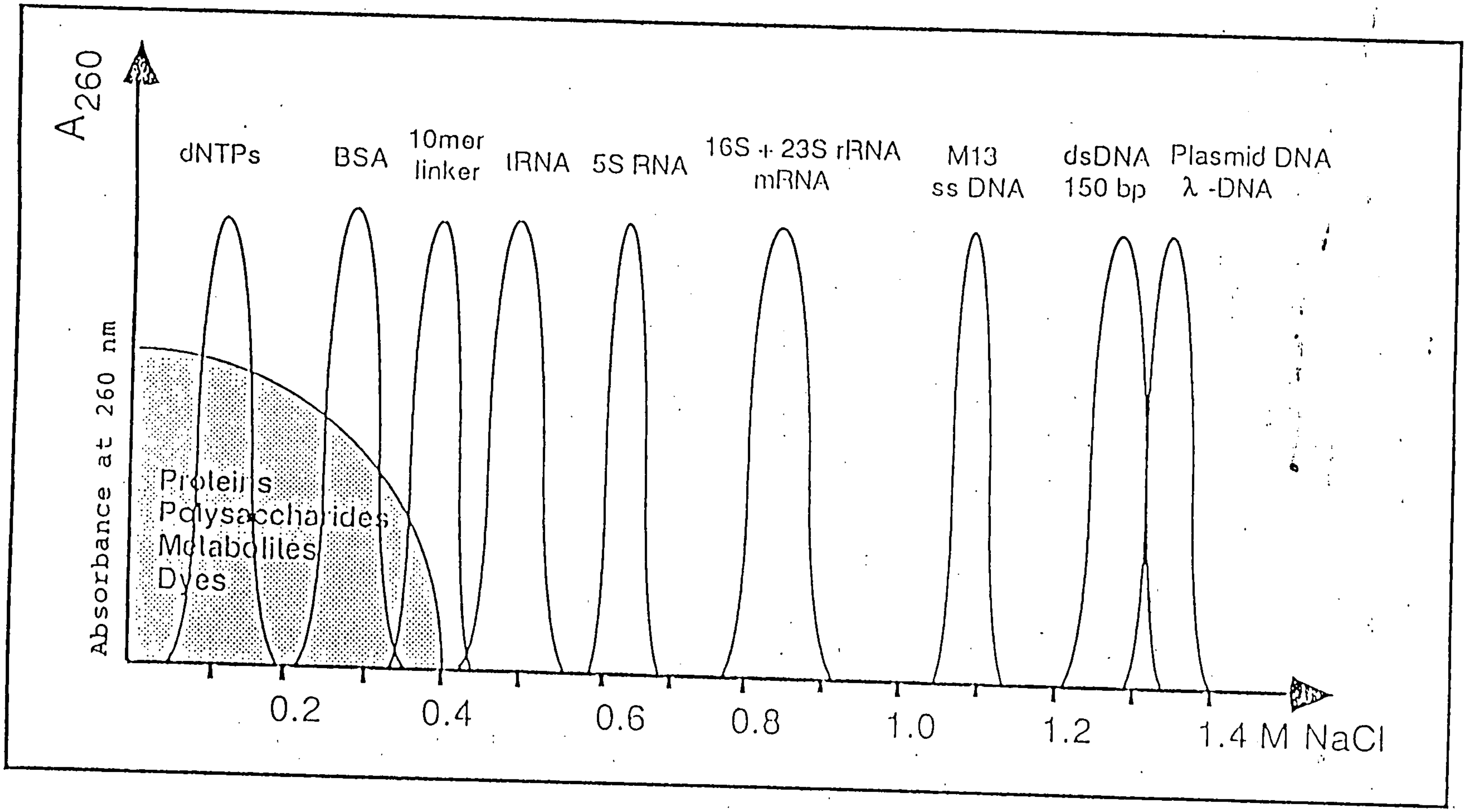



Fig. 4 Elution profiles in NaCl-gradient elution at pH 7.0.